Investigating interactions between epicardial adipose tissue and cardiac myocytes: what can we learn from different approaches?

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Investigating interactions between epicardial adipose tissue and cardiac myocytes: what can we learn from different approaches?

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Abstract

Heart disease is a major cause of morbidity and mortality throughout the world. Some cardiovascular conditions can be modulated by lifestyle factors such as increased exercise or a healthier diet, but many require surgical or pharmacological interventions for their management. More targeted and less invasive therapies would be beneficial. Recently it has become apparent that epicardial adipose tissue plays an important role in normal and pathological cardiac function, and it is now the focus of considerable research. Epicardial adipose tissue can be studied by imaging of various kinds, and these approaches have yielded much useful information. However at a molecular level it is more difficult to study as it is relatively scarce in animal models and, for practical and ethical reasons, not always available in sufficient quantities from patients. What is needed is a robust model system in which the interactions between epicardial adipocytes and cardiac myocytes can be studied, and physiologically relevant manipulations performed. There are drawbacks to conventional culture methods, not least the difficulty of culturing both cardiac myocytes and adipocytes, each of which has special requirements. We discuss the benefits of a three-dimensional co-culture model in which *in vivo* interactions can be replicated.

Non-Standard abbreviations

2D	2 dimensional
3D	3-dimensional
CAD	coronary artery disease
CT	computer tomography
EAT	epicardial adipose tissue
EC-coupling	excitation-contraction coupling
FABP4	fatty-acid binding protein 4
FDG-PET	fluoro-deoxyglucose PET
FFA	free fatty acid
MRI	magnetic resonance imaging
PET-CT	positron-emission computer tomography
RyR	ryanodine receptor
SAT	superficial adipose tissue
SR	sarcoplasmic reticulum
TAG	triacylglycerols

Introduction

The mammalian heart is surrounded by layers of visceral fat known as paracardial adipose tissue. The outermost layer of adipose tissue, the pericardial layer, can cover the entire structure and is separated from the surface of the heart by a layer of connective tissue, the fascia. Beneath this fascia, however, is another layer of fat that is directly apposed onto the heart, with no physical barrier between the two: the epicardial adipose tissue (EAT, see figure 1). EAT is located primarily around cardiac blood vessels, in the atrio-ventricular cleft, and around the right ventricle; it can also extend into the myocardium and this is not in itself indicative of disease (Rabkin, 2007). Post-mortem studies have shown that EAT can cover 80% of the heart and contribute up to 20% of ventricular mass in normal individuals (Rabkin, 2007; Chechi *et al.*, 2015). Epicardial adipocytes have many characteristics of white adipocytes, but produce UCP-1, a marker characteristic of brown adipocytes (Nicholls *et al.*, 1978; Sacks *et al.*, 2009; Sacks *et al.*, 2013). Thus some authors have described this tissue as 'beige' or 'brite' (Gaborit *et al.*, 2013; Chechi *et al.*, 2015).

The functional relationship between the heart and the overlying EAT is important; indeed the adipocytes within EAT, and their products, are critical to the function of the heart. Epicardial adipocytes are an essential source of free fatty acids, which cardiac myocytes preferentially utilise for energy (Marchington *et al.*, 1989). EAT also provides thermal and physical protection for the heart (Rabkin, 2007; Chechi *et al.*, 2015), and, importantly, is a rich source of adipokines which can interact with cardiac myocytes and other tissues in a paracrine manner to promote efficient cardiac function (Talman *et al.*, 2014). However, these beneficial functions of EAT can be diminished or negated by the release of signals that evoke pathological changes in coronary blood vessels and cardiac myocytes. Thus there is interest in investigating interactions between EAT and the heart. Such studies could identify

clinically relevant processes or molecules that might allow pharmacological interventions for a range of cardiovascular diseases.

Considerable work has shown that adipose tissue is biochemically specialised in a site-specific way relating to its function (MacQueen *et al.*, 2000; Mattacks *et al.*, 2004; Westcott *et al.*, 2006; Pond, 2009). There are clear and consistent differences in the triacylglycerol fatty acids found in adipocytes from different anatomical locations, even within the same adipose depot. Therefore it is not surprising that patterns of gene expression and proteins secreted also differ between adipocytes in a site-specific way. Zdychova *et al.* (2014) studied the secretome of adipocytes explanted and cultured from EAT, renal adipose tissue and superficial adipose tissue (SAT). Mazurek *et al.* (2003) were the first to show differences in cytokines secreted by EAT in patients with coronary artery disease (CAD), and found a higher number of pro-inflammatory cytokines secreted by the EAT. Compared to SAT, the transcriptome of the EAT is typical of that from an inflammatory tissue (McAninch *et al.*, 2015). Not only are the secretome and transcriptome different between SAT and EAT: Gaborit *et al.* (2015) have recently characterised molecular differences between various regions of the EAT (peri-ventricular, peri-coronary and peri-atrial). In general, pathological conditions such as CAD result in a downregulation of genes involved in intracellular trafficking, proliferation and protein catabolism, and an upregulation of genes involved in inflammatory and immune responses.

The structure and function of EAT

Epicardial fat is present from birth in humans and its mass increases with age until between 20 and 40 years of age after which time it becomes independent of age (Rabkin, 2007). It is a dynamic fat depot, and can increase, or remodel, in conditions such as obesity and

inflammation (Hassan *et al.*, 2012; Iacobellis, 2015; Kusayama *et al.*, 2015; McAninch *et al.*, 2015; Matloch *et al.*, 2016; Samanta *et al.*, 2016). During development EAT arises from the splanchnopleural mesodermal lineage, the same origin as mesenteric and omental fat (Marchington *et al.*, 1989; Gaborit *et al.*, 2013), and it therefore shares the same lineage as cardiac myocytes (Martinsen *et al.*, 2005). The two tissues are closely related in that they share circulation and innervation (Marchington *et al.*, 1989; Arora *et al.*, 2003). Intrinsic cardiac neurons and ganglionated plexuses, containing adrenergic and cholinergic neurons, are embedded in EAT (Arora *et al.*, 2003; White, 2016). Zhou *et al.* (2014) showed that the cardiac ganglionated plexus in EAT incorporates the autonomic innervation (both sympathetic and parasympathetic) of the heart, and affects atrial function. Moreover, Balcioglu *et al.* (2015) found that a sympathovagal imbalance, a predictor of arrhythmia, was associated with EAT thickness. Parisi *et al.* (2016) also found that in patients with heart failure there was cardiac sympathetic denervation that could be correlated with increased epicardial adipocyte volume. EAT is not just a passive supporting structure for cardiac innervation, but is innervated in its own right. Zeng *et al.* (2015) have shown, although not in epicardial adipocytes, that sympathetic innervation of adipose tissue mediates the lipolytic action of leptin. Adipocytes from many sources have been shown to be sensitive to both β- and α- adrenergic stimulation, which directly increase lipolysis (Mattacks *et al.*, 1999; Mattacks *et al.*, 2005) thus providing an increased supply of free fatty acids for the heart's energy requirements (see above).

The thickness of EAT is a predictor for several types of cardiovascular disease signs such as stenosis, calcification, atheroma, stiffness, plaque development and arrhythmias including atrial fibrillation (Mookadam *et al.*, 2010; Hassan *et al.*, 2012; Gaborit *et al.*, 2013; Hatem *et al.*, 2014; Furuhashi *et al.*, 2016; Siegel-Axel *et al.*, 2016; Wong *et al.*, 2016). Moreover

increased EAT volume is associated with a lengthened PR interval (Hung *et al.*, 2015), suggesting that EAT can interfere with electrical conduction through the heart. Recently cognitive decline and dementia have also been linked to an increased EAT thickness (Mazzoccoli *et al.*, 2014; Viscogliosi *et al.*, 2016). However, EAT thickness varies between ethnicities, so no specific threshold value for clinical significance has yet been established (Willens *et al.*, 2008; Pierdomenico *et al.*, 2013; Yañez-Rivera *et al.*, 2014). The relative amount of EAT varies between species. In general, larger mammals have more prominent EAT than smaller animals like rodents (Marchington *et al.*, 1989). Recent studies have shown that mice possess a limited amount of EAT, located in the atrio-ventricular groove (Yamaguchi *et al.*, 2015). Nevertheless it is difficult to obtain large amounts of EAT from rodents, limiting their use as a source of tissue for *ex vivo* and *in vitro* investigations.

In common with other visceral fat depots, EAT has a function in lipid trafficking, and its mass is correlated with whole-body adiposity (Iacobellis *et al.*, 2004). However, the mean adipocyte volume is around half the value found in other visceral adipose depots (Marchington *et al.*, 1989) suggesting that the primary function of epicardial adipocytes is not fat storage. Moreover, even though the adipocytes in EAT can take up fatty acids from the circulation, convert them to triacylglycerols (TAG), and subsequently lipolyse and release them, these processes are not regulated by insulin in the same way as in adipocytes from other depots (Burgeiro *et al.*, 2016): epicardial adipocytes are specialised for their role supporting heart muscle (Marchington *et al.*, 1990). EAT is not simply a benign store of excess fat, since it is metabolically active and interacts with surrounding tissues, including the contractile cardiac myocytes within the heart (Gaborit *et al.*, 2013). It is this last point that is the focus of our work, as we seek to understand how the metabolic activities of epicardial adipocytes support, or in pathological conditions dysregulate, cardiac myocyte function. The

metabolic cross-talk between EAT and the myocardium is poorly understood; nevertheless there is compelling evidence that it exists and is important in health and disease. Glucose uptake and lipid metabolism in EAT have been shown to be impaired in patients with heart failure, and these effects are modulated in patients who also had diabetes (Burgeiro *et al.*, 2016). There is a growing body of evidence of changes in gene expression in EAT that can be linked to cardiovascular disease (Salgado-Somoza *et al.*, 2012; Agra *et al.*, 2014a; Agra *et al.*, 2014b). This topic is extensive and has recently been systematically reviewed by Maghbooli *et al.* (2015). These authors concluded that the 'hub' genes for EAT involvement in cardiovascular disease were IL6 and p53; the latter gene was also identified as of major importance in coronary artery disease (Agra *et al.*, 2014b).

Like other cells that have a high demand for fatty acids (such as T cells containing a Gall body) cardiac myocytes contain fat droplets (Iozzo, 2011). It is thought that this sequestration of fatty acids allows them to be used rapidly when a physiological need arises. The particular mix of fatty acids sequestered varies with cell type and anatomical site (MacQueen *et al.*, 2000; Pezeshkian *et al.*, 2009; Pond, 2009), and it would be interesting to measure the particular fatty acid mix found within cardiac myocytes and compare it with the fatty acid profiles of EAT. Burgeiro *et al.* (2016) reported that the fatty acid composition of epicardial adipocytes is altered in patients with heart failure and diabetes, and this might suggest the existence of related changes in the fatty acid composition of cardiac myocytes in pathological states.

Overview of adipokines

It is well known that adipocytes secrete signalling molecules, and other factors that affect the cardiovascular system. Adipokines are frequently measured in the general circulation

(reviewed by Iacobellis, 2015) but these measurements cannot address the paracrine interactions that take place at a local level. It is not yet clear which of the cohort of paracrine messengers cause particular pathological changes in cardiac myocytes. Moreover, it has been demonstrated that the secretome of adipocytes can change under pathological conditions such as inflammation, thereby promoting the release of harmful signals (Kusayama *et al.*, 2015; Venteclef *et al.*, 2015; Furuhashi *et al.*, 2016; Iacobellis, 2016). Adipokines have been linked to many pathologies (Deng *et al.*, 2010b) but the mechanisms involved remain to be elucidated.

The *in vivo* effects of adipokines are complex and not easily predictable. For example, the effects can be dose- or concentration-dependent, or may only become obvious if a particular combination of adipokines is present. As an example, the cardioprotective effect of omentin-1 only becomes obvious when cardiac function is impaired, such as in diabetes mellitus (Greulich *et al.*, 2013). Interestingly, the baseline adiponectin concentration in EAT is lower than that found in other fat depots (Elie *et al.*, 2016), yet adiponectin can partially rescue the diminished cardiac contraction observed in obese mice (Dong *et al.*, 2009). Both omentin-1 and adiponectin are considered to be cardioprotective not only because of their direct effects on cardiac myocytes, but also because they reduce inflammation, improve endothelial function, and reduce oxidative stress (Gaborit *et al.*, 2015; Matloch *et al.*, 2016). The cardioprotective function of adrenomedullin is thought to originate in its vasodilative, anti-inflammatory, and anti-oxidative effects, and in its inhibition of hypertrophic remodelling in a disease situation (Silaghi *et al.*, 2007; Fosshaug *et al.*, 2015). Some adipokines, such as resistin, activin A and FABP4 (Lamounier-Zepter *et al.*, 2009; Look *et al.*, 2011; Venteclef *et al.*, 2015) have detrimental effects, whilst others, such as leptin and apelin, can show either helpful or deleterious effects depending on their concentration (Look *et al.*, 2011; Ghantous

et al., 2015). As well as acting on myocytes, some adipokines have been shown to have an effect on cardiovascular endothelia: for example orosomucoid can increase proliferation in endothelial cells and therefore promotes healing of damaged endothelia (Fandino-Vaquero *et al.*, 2014). It is unclear what provokes changes in the secretome of EAT to tip the balance between release of deleterious versus beneficial signals. However Fernandez-Trasancos *et al.* (2016) showed *in vitro* that inflammatory conditions, and glucose levels, affected adiponectin expression in EAT. The reported effects of a number of adipokines are summarised in Table 1.

EATing for a healthy heart

EAT is critical for the function of a healthy heart by supplying free fatty acids (FFA) as the preferred energy source for cardiac myocytes, and by acting as a sink in case of an excess of circulating FFA. Nevertheless, much research has established that through its secretion of pro-inflammatory adipokines EAT is significantly associated with coronary artery disease and atherosclerosis (Shimabukuro *et al.*, 2013; Talman *et al.*, 2014; Furuhashi *et al.*, 2016). In fact, EAT around the coronary vessels can co-localise with areas of plaque development (Prati *et al.*, 2003; Alexopoulos *et al.*, 2010). Peri-coronary EAT shows an upregulation of genes regulating sphingosine metabolism, suggesting a role for EAT in atherogenesis (Gaborit *et al.*, 2015). While it is clear that compromised blood supply can interfere with heart function, our interest lies more with possible direct effects of EAT and its adipokines on the cardiac myocytes, and the disruption to regular beating that can result in arrhythmias such as atrial fibrillation.

As noted above, EAT is a dynamic depot and its size is positively correlated with whole-body fat and obesity. Interestingly, changes in the structure and function of cardiac myocytes, and

the pro-inflammatory status of the adipocytes caused by a high fat diet can precede the onset of weight gain and obesity (Goncalves *et al.*, 2016), and during weight loss protocols EAT reduces in thickness before abdominal fat is reduced (Iacobellis, 2015). This suggests that EAT activity is highly sensitive to nutritional status, and that epicardial adipocytes may act as a sentinel for cardiac dysfunction.

Throughout a typical human lifetime the heart beats 2 million times (Bers, 2002). The contraction of the heart is triggered by action potentials that propagate from the sino-atrial node in the right atrial chamber. As an action potential sweeps across the atrial and ventricular chambers it causes the membrane to depolarise, thus opening voltage-operated calcium (Ca^{2+}) channels and causing a Ca^{2+} influx into the cell. Within the cell, this Ca^{2+} signal is amplified by Ca^{2+}-induced Ca^{2+} release via ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR), thus causing a global Ca^{2+} signal that triggers the intracellular contractile machinery to engage, so that the cells shorten and generate the force to propel blood to the lungs and body. This process is often referred to as excitation-contraction coupling (EC-coupling; Berridge, 2003; Dobrzynski *et al.*, 2013) and is illustrated in Figure 2.

For a regular heartbeat, each action potential must cause a rapid, transient, Ca^{2+} rise within every cardiac myocyte. It is believed that increased automaticity (spontaneous depolarisation of myocytes), acute triggered activity (spontaneous electrical events following recovery from an action potential) or re-entry circuits (return of an electrical impulse to cardiac cells following a refractory period) contribute to the development of arrhythmia. The underlying causes of all these pro-arrhythmic conditions are not fully understood, but substantial evidence has implicated a higher incidence of spontaneous Ca^{2+} signals as a likely cause

(Bers, 2008; Heijman *et al.*, 2012; Voigt *et al.*, 2012). Generally, pro-arrhythmic processes can be classified into electrical and structural remodelling. Electrical remodelling encompasses changes in the ion channel expression, changing the myocyte's response to action potentials and leading to automaticity and triggered activity. Electrical remodelling can occur very rapidly, and is usually reversible. Recent evidence suggests that FFA can cause electrical remodelling in the atria (O'Connell *et al.*, 2015). In contrast, structural remodelling takes longer to develop, and is usually irreversible. The most common structural remodelling process is fibrosis, the formation of fibrotic tissue between cardiac myocytes. As a consequence of fibrosis, the propagation of electrical signals is disrupted because of the presence of non-conducting tissue. Instead of propagating into the next cell, electrical signals spread sideways or backwards and can enter the same cell again, leading to the development of perpetuating rotors of electrical signals, and consequently to arrhythmia (Eisner, 2014; Heijman *et al.*, 2014; Lip *et al.*, 2016). Of interest here is the fatty infiltration between the cardiac myocytes, seen when excess EAT is present, that can transform into fibrotic tissue; adipokines and cytokines secreted from EAT can induce fibrosis (Haemers *et al.*, 2015; Venteclef *et al.*, 2015) and alter the Ca^{2+} handling, and thus the contractions, of cardiac myocytes (Hatem *et al.*, 2016). It appears that the correct functioning of EAT is essential for effective cardiovascular activity, and the interactions between EAT and cardiac myocytes represent an important therapeutic target.

Approaches to studying EAT

EAT has historically been a somewhat neglected tissue (Rabkin, 2007) but the advent of techniques such as echocardiography made it easy to identify and quantify. Extensive work by Iacobellis and others (Iacobellis *et al.*, 2003; Iacobellis *et al.*, 2005; Iacobellis *et al.*, 2009b; Iacobellis *et al.*, 2009a; McAninch *et al.*, 2015; Elisha *et al.*, 2016) showed that EAT

varies between individuals in thickness and volume, and the thickness can be correlated with disease states. For example, there is significantly more EAT in subjects with metabolic syndrome or coronary artery disease than in those without these conditions (Sade *et al.*, 2009; Pierdomenico *et al.*, 2013). The advantage of echocardiography is that it is non-invasive (though it may be coupled with more invasive techniques such as angiography), relatively cheap and widely available (Parisi *et al.*, 2016); its disadvantage is that the studies are observational, giving a 'snapshot' of patients who have already presented with a suspected or actual cardiopathology. Also, using this technique it can be difficult to define the border between epicardial and pericardial fat, which can potentially generate misleading data (Hatem *et al.*, 2016). Other fat depots which would be useful as controls are located in different parts of the body, so the appropriate controls can usually not be obtained from the same patient due to the increased risks and length of the necessary surgery (Fain *et al.*, 2010; Gaborit *et al.*, 2015). One of the few prospective studies undertaken so far reported a correlation of peri-atrial EAT thickness with the future onset of AF in patients who did not show any signs of AF at the time of the scan (Nakanishi *et al.*, 2012). The reproducibility of echocardiographic analysis and interpretation has been questioned (Wong *et al.*, 2016); nevertheless good correlation between echocardiography and other techniques for measuring EAT thickness in specific areas has been reported (Song do *et al.*, 2015).

Other types of imaging studies have been undertaken, such as computed tomography (CT) (Sarin *et al.*, 2008; den Dekker *et al.*, 2014; Harada *et al.*, 2014; Hung *et al.*, 2015; Kitagawa *et al.*, 2015), positron-emission CT (PET-CT) (Janik *et al.*, 2010; Bakkum *et al.*, 2015), magnetic resonance imaging (MRI) (Kim *et al.*, 2012; Gaborit *et al.*, 2013; Hua *et al.*, 2014) and FDG-PET (fluoro-deoxyglucose PET) (Kaushik *et al.*, 2014; Mazurek *et al.*, 2014). The principal advantage of all these methods is that they are non-invasive, and there is generally

much better resolution than in echocardiographic studies. Three-dimensional and volume measurements, rather than simply thickness, are possible: both CT and MRI can specifically measure peri-coronary EAT, which is an important correlate of atherosclerosis (Iacobellis, 2016; Wong *et al.*, 2016). CT studies have confirmed an association between atherosclerosis, myocardial infarction and EAT (Mahabadi *et al.*, 2013; Mahabadi *et al.*, 2014). However CT uses radiation, so is not suitable for follow-up studies requiring repeated measurements. The main drawback of these techniques is that they require expensive equipment and are neither cheap nor easy to access. This is particularly the case for cardiac MRI, regarded as the gold standard because it gives good 3D imaging, does not use radiation, and is the only method to have been verified *ex vivo.* MRI can reliably detect fibrosis and fat depots (Wong *et al.*, 2016). Therefore there is a trade-off between availability and accuracy when considering imaging methods.

Post-mortem examination of patients who have died from other causes can provide some indications of the situation in 'normal' (albeit dead) individuals (Gho *et al.*, 2014; Furuhashi *et al.*, 2016). A drawback to post-mortem studies is that the condition that killed the individual may itself have affected adipose tissue distribution, giving some uncertainty as to what actually constitutes 'normal'. Even when samples can be obtained from live patients, they are usually small and may allow only the analysis of mRNA levels, not the actual expressed protein levels. For ethical reasons it is currently difficult to obtain control patients, although this may change with the advent of large-scale prospective studies.

Going beyond the identification and quantitation of EAT, many studies of the depot have involved molecular and biochemical studies using either blood or tissue biopsies, obtained during elective cardiovascular surgery procedures. These studies have variously used ELISA (Mazurek *et al.*, 2003; Gao *et al.*, 2011; Sacks *et al.*, 2011; Teijeira-Fernandez *et al.*, 2011;

Fosshaug *et al.*, 2015; Venteclef *et al.*, 2015; Elie *et al.*, 2016), PCR (Mazurek *et al.*, 2003; Fain *et al.*, 2010; Gao *et al.*, 2011; Teijeira-Fernandez *et al.*, 2011; Fosshaug *et al.*, 2015), miR analysis (Vacca *et al.*, 2016), transcriptome and secretome analysis (Guauque-Olarte *et al.*, 2011; Imoto-Tsubakimoto *et al.*, 2013; Gaborit *et al.*, 2015; McAninch *et al.*, 2015; Venteclef *et al.*, 2015) to show that the molecular profile of EAT is different from that of other adipose depots, and can change during physiological challenges affecting the heart. This large body of data supports the site-specific, localised role played by EAT, but does not elucidate the question of whether the observed changes are the cause or the effect of physiological disturbance. In order to get around this problem a model system amenable to experimental manipulation is required. Although clinicians are frequently unwilling to translate data derived from animal studies to humans, it remains the case that whole animals, or cells derived from them, constitute a rich source of information and data, and can frequently provide valid experimental models. We discuss some of the options below.

Studying EAT-cardiac myocyte interactions using animal models

Some of the earliest studies of EAT (Marchington *et al.*, 1989; Marchington *et al.*, 1990) used short-term cultures of tissue explants from rodents to study the biochemistry and fat trafficking in epicardial adipocytes. Animal tissue has also been cultured for this purpose by Greulich *et al.* (2011) (guinea pigs and rats), Chilukoti *et al.* (2015) (pigs and rats), and Lage *et al.* (2015) (neonatal rats and cell lines). The availability of a range of mouse knock-out strains has also been exploited by Nevelsteen *et al.* (2013), studying cardiac myocyte function and obesity, though not specifically EAT, and by Castro *et al.* (2015). This group reported on a wide-ranging project in which they studied the metabolomics by MS-NMR and looked at circadian rhythms of brown and white adipose tissue.

In an attempt to elucidate the role of adipokines in cardiac dysfunction, many *in vitro* studies have added purified adipokines to cardiac myocytes in culture, and measured the effects on their function. A drawback of these studies is that the adipokine effects may be complex and are often concentration-dependent (see above): for example the cardioprotective effect of omentin-1 is only evident when applied in combination with inflammatory interleukins (Greulich *et al.*, 2013). It is not easy to replicate in culture relevant physiological conditions, in which there may be subtle differences in adipokine and other cytokine concentrations. One approach to resolving this problem has been to use *ex vivo* cultured EAT biopsy samples to prepare conditioned media for adding to established cultures of (usually) rodent cardiac myocytes. Such an approach has been used to analyse the secretome, and to test for structural and functional effects of the EAT on the cardiac myocytes. Such studies identified activin A as the major factor causing fibrosis (Venteclef *et al.*, 2015).

It is known that both a high fat diet and diabetes mellitus cause deterioration in heart function. Medium conditioned by the EAT of animals eating a high fat diet recapitulated these effects (reduced contraction strength and Ca^{2+} amplitude in isolated rat cardiac myocytes), whilst medium conditioned by SAT did not (Greulich *et al.*, 2011). In a later study, the authors found that the reduced cardiac function was caused by a lack of omentin-1, which in healthy animals protects the cardiac myocytes from the detrimental effects of cytokines like interleukins. Omentin-1 cannot fulfil this function when its levels are reduced in diabetes mellitus type 2 (Greulich *et al.*, 2013). Other cardioprotective effects of omentin-1 have been mentioned in Table 1. Another adipokine that impairs cardiac function, specifically the cardiac myocyte's contractility, is FABP4, which was identified and its effects characterised by Lamounier-Zepter *et al.* (2009 and 2014). The detrimental effects of FABP4

are potentiated in the presence of epoxyeicosatrienoic acids, which are also secreted by the EAT.

Although many such studies have elucidated the role of EAT and related it to cardiac function, little has yet been done to elucidate the physiological interplay between these tissues. In order to do this rigorously a culture methodology that is robust, reproducible and physiologically relevant must be adopted. Conventional two-dimensional (2D) culture can be, and has been, used but although it offers the opportunity for extensive experimental manipulations it is a poor representation of the *in vivo* physiological situation. Whilst a cardiac myocyte culture, using neonatal cells, is relatively straightforward to set up, it is difficult to mimic any effects of neighbouring adipocytes. Using adipocyte-conditioned media, as described above, means that cardiac myocytes are presented with a bolus of adipocyte-derived molecules, which, although more representative than the addition of isolated adipokines, are nevertheless likely to be at different concentrations from those present in the paracrine flow that occurs *in vivo*. In addition, labile molecules will not be adequately represented in conditioned medium, so their effects can be missed. From a clinical perspective it is necessary to find treatments that allow the long-term management of adipocyte-cardiac myocyte interactions. Acute responses evoked by bolus additions of adipocyte-conditioned medium can pinpoint factors that affect cardiac myocyte function, but it is not a method that allows prolonged study, and management, of cardiac myocyte functionality and viability in the presence of persistent paracrine signals. For these reasons, we consider that a better method for studying interactions between cell types may be to use three-dimensional (3D) co-cultures, and we elaborate on this below.

The 3D approach

Primary adipocytes are difficult to work with *in vitro*, as they are very fragile. They cannot be retained in 2D cultures because their buoyant density decreases as they accumulate triacylglycerols, causing them to detach from the culture substrate and become lost during medium changes. Furthermore, only cardiac myocytes prepared from neonatal rodents can be kept in culture for prolonged periods of time, as cardiac myocytes isolated from adult animals de-differentiate quickly after being isolated and placed in 2D culture. However in 3D culture it is possible to keep cardiac myocytes differentiated and spontaneously beating for a period of up to 6 weeks (Burridge *et al.*, 2014). Their phenotype resembles that of a mature differentiated myocyte when they are kept in a 3D culture that has a stiffness mimicking that of the heart (Engler *et al.*, 2008; Pontes Soares *et al.*, 2012; Bian *et al.*, 2014). Similarly, adipocytes show good survival in 3D cultures, and retain the *in vivo* structural and functional characteristics of mature adipocytes (Daya *et al.*, 2007). Because collagen gels are translucent, 3D co-cultures are amenable to microscopic analyses that allow real-time measurement of cellular responses and phenotype. Contractions of the whole gel or of cells inside the gel can be measured by force transducers or by visualising cell contraction, and intracellular Ca^{2+} changes can be monitored using fluorescent Ca^{2+} sensitive dyes (Shapira-Schweitzer *et al.*, 2009; Ye, 2011; Bian *et al.*, 2014; Chiu *et al.*, 2014). Furthermore, the culture supernatants can be assayed for secreted molecules, and the two cell types can be independently recovered for proteomic, genomic and molecular analysis (Daya *et al.*, 2007; Phillips *et al.*, 2011; Georgiou *et al.*, 2015). Thus primary cardiac myocytes and adipocytes can be co-cultured for prolonged periods of time, allowing paracrine signalling to occur, and the interactions between these cells to become established in a way that closely models the *in vivo* situation. The functionality and viability of cells within the 3D collagen matrix can be closely monitored, and both cell types can be recovered separately for subsequent *in vitro* molecular analysis. By culturing adipocytes and cardiac myocytes within a 3D co-culture

model we can better mimic the normal concentrations and rates of paracrine delivery of signals between these cells.

Conclusions

Epicardial adipose tissue is now well established as an important modulator of cardiac function. Changes in EAT metabolism can be associated with a number of pathologies both in human patients and in animal models, and it seems clear that this relationship is a strong candidate for therapeutic intervention. However the relationship is a complex one: not only does the particular mix of paracrine signals vary with physiological state, but the anatomical location within the EAT appears to play a part as well. Like all adipose tissue, EAT demonstrates site-specific functionality.

Although there have been many important contributions towards elucidating the interplay between cardiac myocytes and EAT, more questions remain unanswered. Key to identifying the underlying mechanism(s) is the deployment of appropriate experimental techniques that will allow experimental manipulation of the relationship over the short and medium term. Only then will it be apparent whether the changes seen in EAT metabolic activities are the cause or the result of the pathologies. Current technologies range from the 'snapshots' provided by imaging and analysis of biopsy samples from patients through to highly reductionist *in vitro* techniques. One limitation of human studies will always be the lack of 'healthy EAT' samples as a control. Our preference is to develop a 3D co-culture system in which both cardiac myocytes and adipocytes can flourish together over several weeks, and in which experimental modifications and subsequent analysis are straightforward.

Author contributions

The authors contributed equally to this work.

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Conflicts of interest

The authors declare no conflicts of interest.

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Table 1 Cardiovascular effects of adipocytokines secreted by EAT. This table summarises publications describing effects of adipokines secreted from EAT. +, positive effects; ±, positive and negative effects; -, negative effects on cardiovascular function. [Please also see separate document for the table in landscape format, our preferred formatting option.]

Name	Observation	Methodology	Overall effect	Reference
General inflammatory adipokines	EAT secretes pro-inflammatory cytokines (TNFα, MCP1, IL-1, IL-1β, IL-1 Ra, IL-6, IL-8, PAI-1, CRP, haptoglobin) which are all linked to coronary artery disease (CAD). NFκB upregulated in inflammatory states	Review articles.	-	(Mazurek *et al.*, 2003) (Gaborit *et al.*, 2013)
Adiponectin	Improves endothelial function via endothelial NO synthase (eNOS) In obesity, reduces oxidative stress, further protecting the endothelium	Isolated aortic tissue from rats on normal *vs*. high fat diet. Adiponectin addition. eNOS and iNOS responses in vascular tissue were characterised. Responses of rat aortic tissue and human aortic endothelial cells to adiponectin addition were characterised (endothelial function, eNOS).	+	(Li *et al.*, 2007) (Deng *et al.*, 2010a)
	Improves the redox state in blood vessels by restoration of eNOS coupling	Human adipose tissue samples (perivascular, subcutaneous and mesothoracic), endothelium from matching vein and arteries. Adiponectin	+	(Margaritis *et al.*, 2013)

		gene expression and vascular responses to eNOS were characterised.		
	Decreased levels in obesity	Abdominal and epicardial adipose tissue from humans. CT study in humans (atherosclerosis patients). Adipokine plasma levels were measured.	-	(Cheng *et al.*, 2008) (Greif *et al.*, 2009)
	Recombinant adiponectin can successfully reverse some harmful effects of EAT-derived factors	EAT samples from human CAD patients ± diabetes. Conditioned medium, effects on THP-1 cells and endothelial cells were characterised.	+	(Karastergiou *et al.*, 2010)
	Generally cardioprotective, downreglated in CAD and heart failure	Review article	±	(Iacobellis, 2015)
	Decreased in CAD Possible negative effects in elderly heart-failure patients	Review article	-	(Gaborit *et al.*, 2013)
	Rescues reduced contractility, reduces the speed of contraction and increases peak Ca^{2+} in cardiac myocytes from $LepR^{db/db}$ mice. No effects on lean control mice	Isolated cardiac myocytes from $LepR^{db/db}$ mice & lean control mice. Responses to adiponectin.	+	(Dong *et al.*, 2009)
	Reduced secretion in presence of macrophages, except in the presence of EPA	*In vitro* co-culture of adipocytes and macrophages from *ob/ob* mice, high fat diet and control mice; EPA treatment. Adiponectin concentration in human plasma from obese patients, ± EAT.	-	(Itoh *et al*., 2007)
	Anti-hypertrophic	Whole rat hearts, Langendorff prep. Conditioned medium from isolated adipocytes and pure cytokines. Effects on contractility.	+	(Look *et al.*, 2011)
	Inversely correlated with atherosclerosis	Human periaortic, pericoronary and apical EAT samples. Adipokine expression.	+	(Spiroglou *et al.*, 2010)
FABP4	Suppresses cardiac myocyte contractions	Isolated rat cardiac myocytes, FABP4 effects on myocyte function. Isolated rat cardiac myocytes. Adipocyte conditioned medium. Addition of isolated 5,6-EET and FABP4; effects on cardiac function.	-	(Lamounier-Zepter *et al.*, 2009) (Lamounier-Zepter *et al.*, 2014)
	Associated with left ventricular dysfunction in obese subjects	Human obese patients ± left ventricular dysfunction. Adipokine levels and ventricular diastolic function measured.	-	(Baessler *et al.*, 2014)

	Pro-inflammatory	Review article.	-	(Xu *et al.*, 2012)
	Supresses cardiac myocyte function; intracellular uptake of FA and intracellular transport; upregulated in EAT from patients with metabolic syndrome	EAT and ascending aorta tissue samples from human metabolic syndrome patients. FABP4 expression.		(Vural *et al.*, 2008)
FABP3	Increased levels in EAT (but not SAT) from heart failure patients	SAT and EAT biopsies from human heart failure and control patients. Plasma adipokine levels were measured.	-	(Fosshaug *et al.*, 2015)
	Suppresses cardiac myocyte contractions	Isolated rat cardiac myocytes, FABP4 effects on myocyte function were examined.		(Lamounier-Zepter *et al.*, 2009)
Activin A	Pro-fibrotic	Human EAT and SAT biopsies, fibrotic properties on organo-culture models of rat atria.	-	(Venteclef *et al.*, 2015)
	Upregulated in animals eating a high fat diet	EAT and SAT from guinea pigs on high fat vs. normal diet to prepare conditioned media. Tested for their effects on contractile function and insulin secretion in rat cardiac myocytes.	-	(Greulich *et al.*, 2011)
	Positively associated with atrial fibrillation	Editorial Review article	-	(Hatem, 2014) (Hatem *et al.*, 2014)
	Reduces cardiac myocyte contractility by reducing Ca^{2+} flux	EAT biopsies from human diabetes mellitus patients and controls used to prepare conditioned medium. Adipokine measurements and effects on contractile function and insulin secretion in rat cardiac myocytes.	-	(Greulich *et al.*, 2012)
	Increased levels in patients with low left ventricular function	Human EAT and SAT biopsies, fibrotic properties on organo-culture models of rat atria.	-	(Venteclef *et al.*, 2015)
Adreno-medullin	Cardioprotective	EAT and SAT samples from human CAD patients and controls. Adrenomedullin expression was measured.	+	(Silaghi *et al.*, 2007)
	Reduced mRNA and protein levels in CAD	EAT samples from human CAD patients and controls. Adrenomedullin levels were measured.	+	(Iacobellis *et al.*, 2009a)
	Increased mRNA in patients with CAD	Blood, EAT and SAT from human CAD patients and controls. Adipokine levels were measured.	-	(Shibasaki *et al.*, 2010)
	Increased levels in EAT but not SAT from heart failure patients	SAT and EAT biopsies and plasma samples from human heart failure patients and controls. Adipokine levels were measured.		(Fosshaug *et al.*, 2015)

Omentin-1	Anti-inflammatory and promotes insulin sensitivity. Improves endothelial function, and reperfusion after ischaemia, by increasing the production of nitric oxide by eNOS.	Human EAT and SAT samples, gene expression was characterised.	+	(Gaborit *et al.*, 2015)
	Plasma level decreases in obesity and in type 2 diabetes.	EAT biopsies from human diabetes mellitus patients and controls used to create conditioned medium. Omentin-1 levels and effects on contractile function and insulin secretion in rat cardiac myocytes were measured.	+	(Greulich *et al.*, 2013)
	mRNA and protein expression increased in patients with CAD, particularly from EAT around areas of stenosis	Plasma, SAT and EAT samples in human CAD patients and controls. Adipokine levels were measured.		(Du *et al.*, 2016)
Leptin	Reduces endothelial-dependent vasodilation	Rat, leptin effects on blood pressure were characterised.	-	(Bełtowski *et al.*, 2004)
	Increased in obesity	Abdominal and epicardial adipose tissue from human atherosclerosis patients. CT study; plasma levels of adipokines were measured.	-	(Cheng *et al.*, 2008) (Greif *et al.*, 2009)
	Chemo attractive to monocytes	THP-1 macrophages from $LepR^{db/db}$ and $LepR^{+/+}$ mice. Responses to purified leptin and MCP-1 were characterised.	-	(Gruen *et al.*, 2007)
	Decreases high density lipoproteins and apolipoprotein A1 concentrations	Correlation of leptin and HDL levels in healthy humans.	-	(Rainwater *et al.*, 1997)
	Activates C-reactive protein (CRP) and serum amyloid A	Correlation study of BMI and serum adipokine levels in healthy humans.	-	(Kazumi *et al.*, 2003)
	Increases oxidative stress, inflammation and smooth muscle proliferation, recruits monocytes and via macrophages accelerates the formation of foam cells	Review article	-	(Matloch *et al.*, 2016)
	Rapidly induces hypertrophy in cultured neonatal rat ventricular myocytes	Neonatal rat ventricular myocytes. Leptin addition. Measurement of hypertrophy.	-	(Rajapurohitam *et al.*, 2003)
	Increases blood pressure and heart rate via sympathetic nervous system stimulation and causes hypertension	Fasting leptin plasma levels in humans with hypertension were measured.	-	(Paolisso *et al.*, 1999)
	Physiological doses abolish the negative	Rat models of ischemia/reperfusion injury ±	+	(Smith *et al.*, 2010)

	inotropic effects of IL-1β and can protect from reperfusion injury	leptin administration were examined.		
	Reduce endothelial-dependent vasodilation	Rat, leptin effects on blood pressure were measured.	-	(Bełtowski *et al.*, 2004)
Resistin	Circulating levels increased in obesity	Abdominal and epicardial adipose tissue from human atherosclerosis patients. CT study; plasma levels of adipokines were measured.	-	(Cheng *et al.*, 2008) (Greif *et al.*, 2009)
	Promotes cardiac hypertrophy in rodents	Neonatal rat ventricular myocytes and adult rat cardiac myocytes. Resistin overexpression. Cardiac function and signalling pathways were examined.	-	(Kim *et al.*, 2008)
	Impairs endothelial function	Review article	-	(Pang *et al.*, 2006)
	Increased circulating levels in heart failure and myocardial infarction	Human myocardial infarction, ischemic stroke patients and controls. Plasma resistin levels were measured. Human HF patients, adipokine levels in blood samples were measured.	-	(Weikert *et al.*, 2008) (Frankel *et al.*, 2009)
	Associated with atherosclerosis, myocardial infarction and coronary artery disease	Adipokine secretion in EAT from human acute coronary syndrome vs. CAD patients. Human myocardial infarction patients. Blood, EAT and SAT samples. Resistin levels were measured.	-	(Langheim *et al.*, 2010) (Rachwalik *et al.*, 2014)
Apelin	Positive inotrophic effect in normal and failing myocardium. Protective against ischemia and reperfusion injury in rats	Rat hearts, pressure overload models and isolated neonatal rat ventricular myocytes. Apelin effects on cardiac function were measured. Ischemic reperfusion injury rat model, effects of apelin addition were examined. Heart failure control rats, cardiac function after apelin addition was characterised.	+	(Szokodi, 2002) (Berry *et al.*, 2004) (Kleinz *et al.*, 2008)
	Positively correlated with obesity; promotes a cardioprotective response	Apelin-receptor KO mice, characterisation of cardiac function.	+	(Scimia *et al.*, 2012)
	Stimulates contractility in cardiac myocytes	Rat hearts, pressure overload models and neonatal rat ventricular myocytes. Apelin effects on cardiac function were examined.	+	(Szokodi, 2002)

	Exogenous apelin has anti-inflammatory effects and reduces the formation of aortic aneurisms	Mouse aortic aneurysm model ± apelin. Adipokine expression in macrophages, smooth muscle cells and fibroblasts was measured.	+	(Leeper *et al.*, 2009)
Vaspin	Correlated with atherosclerosis in a site-specific way	Human periaortic, pericoronary and apical EAT samples. Adipokine expression was measured.	-	(Spiroglou *et al.*, 2010)
	Circulating levels correlated with reduced cardiac flow reserve	Human nonalcoholic fatty liver disease patients. Measurement of EAT thickness, coronary flow reserve and chemerin and vaspin serum levels.	-	(Yilmaz *et al.*, 2011)
Visfatin	Site-specific expression within EAT, increased in patients with atherosclerosis	Human periaortic, pericoronary and apical EAT samples. Adipokine expression was measured.	-	(Spiroglou *et al.*, 2010)
	Proinflammatory; produces endothelial dysfunction	Human diabetes patients and controls. Plasma visfatin levels. Endothelial function was measured.	-	(Takebayashi *et al.*, 2007)
	Exogenous visfatin reduces cell death and myocardial infarct size after reperfusion	Mouse ischemia/reperfusion injury model ± visfatin. Isolated myocytes in hypoxia ± visfatin. Signalling pathways and mitochondrial function were measured.	+	(Lim *et al.*, 2008)
Chemerin	Positive correlation with atherosclerosis	Human periaortic, pericoronary and apical EAT samples. Adipokine expression was measured.	-	(Spiroglou *et al.*, 2010)
	mRNA and protein expression increased in patients with CAD	EAT and SAT and serum samples from human CAD patients and controls. Adipokine levels were measured.	-	(Gao *et al.*, 2011)
	Induces apoptosis in mouse cardiac myocytes in culture	Mouse cardiac myocytes; insulin and TNF-α treatment. CMKLR1 and chemerin expression were measured.	-	(Rodriguez-Penas *et al.*, 2015)
Orosomucoid	At low levels, orosomucoid secretion from EAT protects cultured cardiac myocytes from apoptosis due to hypoxia At high levels orosomucoid is a risk factor for myocardial infarction and heart failure.	EAT samples from human biopsies were used to prepare conditioned medium. Rat cardiac myocyte cell line (H9C2) and neonatal rat ventricular myocytes. Effects on cell viability ± hypoxia were measured.	±	(Lage *et al.*, 2015)
	Increasing endothelial cell proliferation, promotes healing of damaged endothelia	EAT and SAT samples from type 2 diabetes mellitus patients used to prepare conditioned medium. Adipokine secretion after isoproterenol stimulation and effects of conditioned medium measured on endothelial cell function and	+	(Fandino-Vaquero *et al.*, 2014)

		wound healing were measured.		

Ligands		
Leptin	FABP4	Adrenomedullin - human
Adiponectin	FABP3	Adrenomedullin - mouse
Chemerin	5,6-EET	Adrenomedullin - rat

Targets	
Leptin receptor	Apelin receptor
Adipo1 receptor	Chemerin receptor
Adipo2 receptor	

Families
Adiponectin receptor
Fatty acid-binding proteins
Chemerin receptor

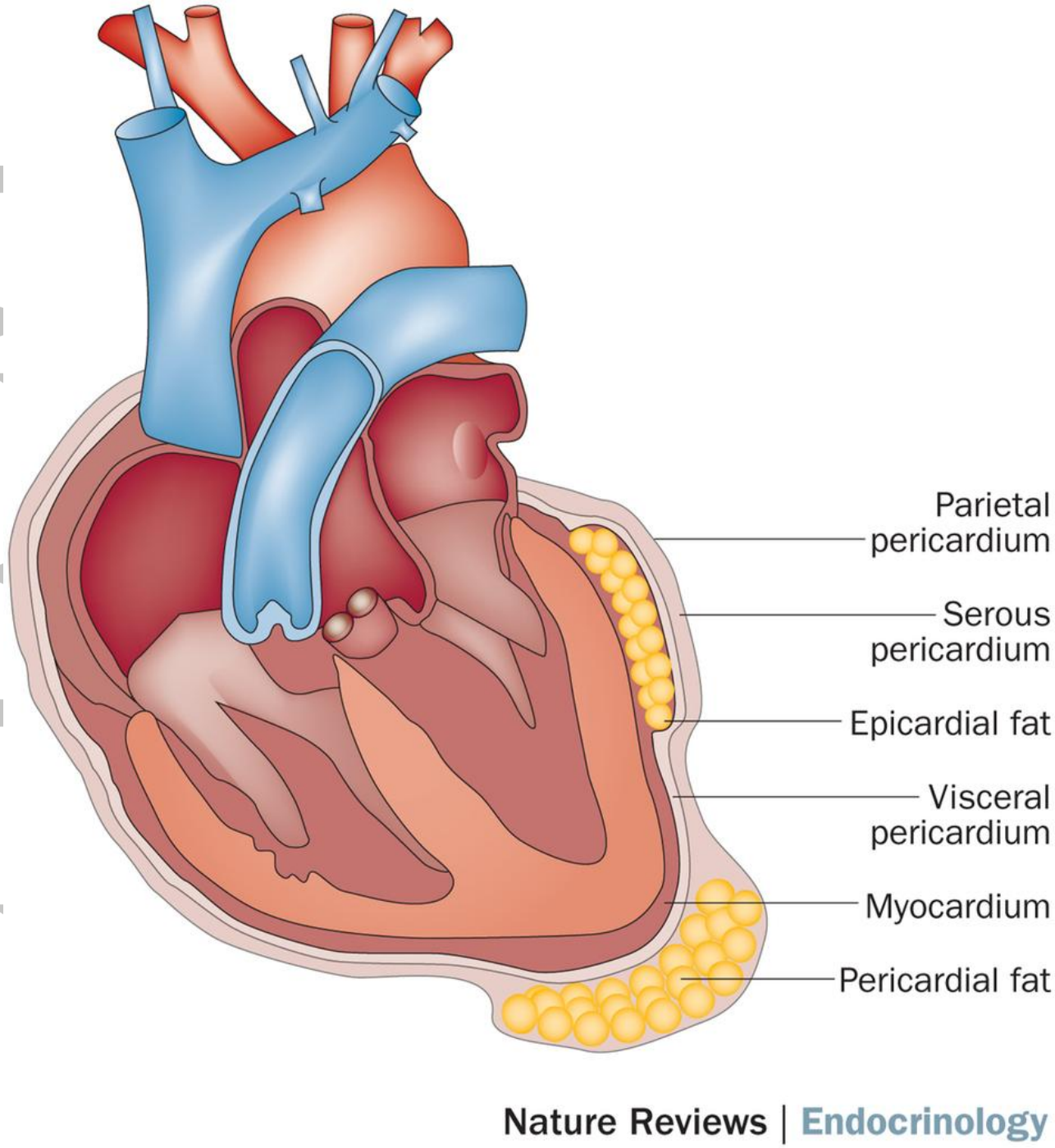

Figure 1 Diagram illustrating the positions of epicardial and pericardial fat depots in the heart. Note the intimate association between the epicardial fat and the myocardium. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Endocrinology. (Iacobellis, G. (2016) Nat Rev Endocrinol 11(6): 363-71). Copyright (2016).

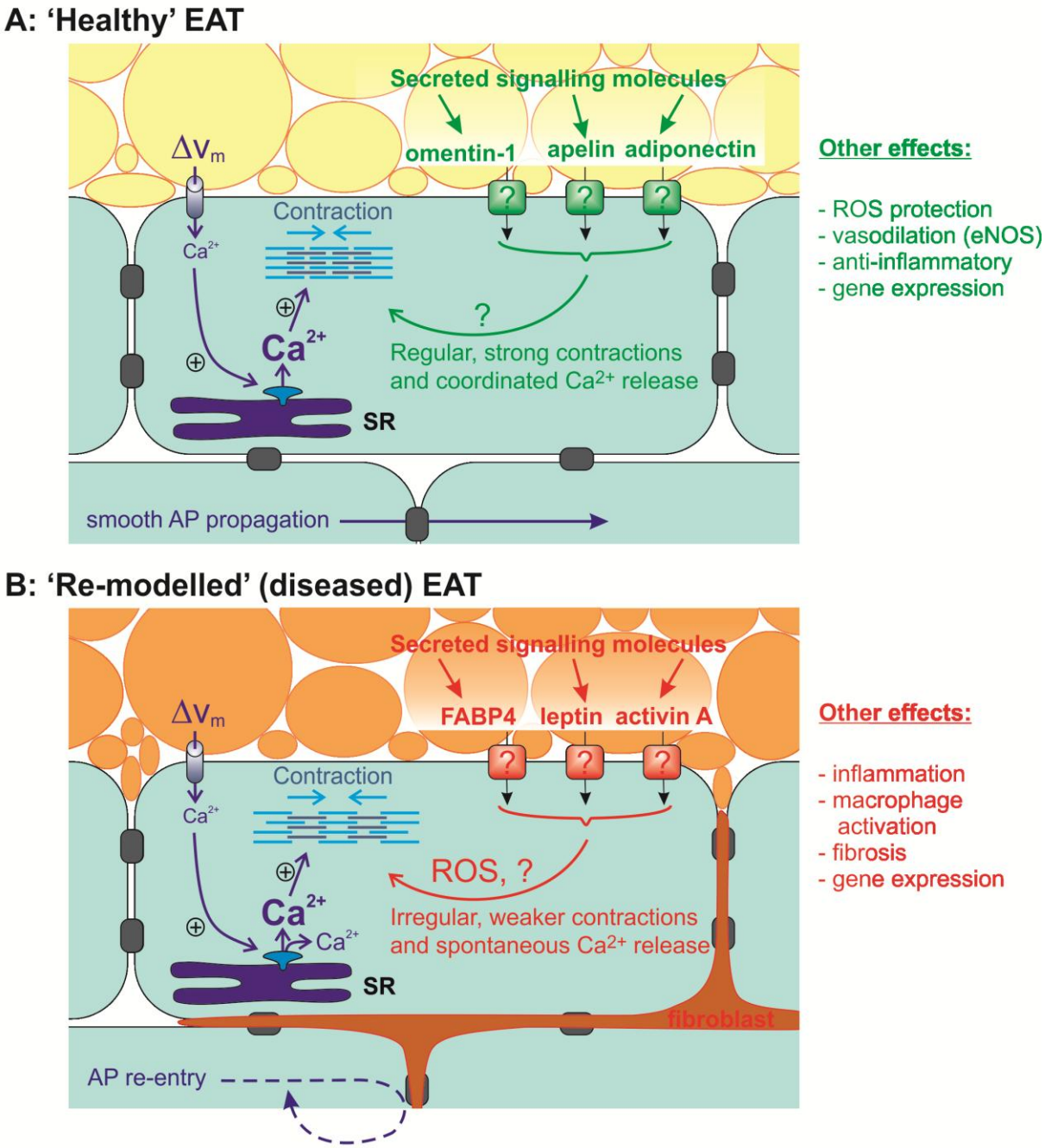

Figure 2 Model of potential interactions between cardiac excitation-contraction coupling and EAT. The figure illustrates places where adipokines might affect the excitation-contraction coupling of cardiac myocytes. Panel A shows the excitation-contraction coupling in cardiac myocytes in a healthy situation. Arrival of an action potential (ΔVM) opens voltage-gated Ca2+ channels in the sarcolemma, causing the influx of Ca2+ ions. The increase in the intracellular Ca2+ concentration activates Ca2+-activated Ca2+-

release via ryanodine receptors localised on the sarcoplasmic reticulum (SR), causing the myocyte to contract. Adipocytes in the EAT are in contact with the cardiac myocytes, and can reach between them. They secrete e.g. omentin-1, apelin and adiponectin, which act on the cardiac myocytes in a positive manner, sustaining their regular contractions. They also affect processes other than the myocyte's contraction, e.g. gene expression, anti-inflammatory functions, and protection from reactive oxygen species (ROS). Panel B shows changes occurring in a disease situation, where the secretome from the EAT is remodelled. Adipocytes infiltrate deeper between the myocytes and release different adipokines, e.g. activin A, leptin and FABP4. Activin A activates fibrosis, which in turn hinders the smooth propagation of action potentials and can cause pro-arrhythmic re-entry circuits. FABP4 and apelin reduce the myocyte's contraction strength. Adipokines can increase intracellular ROS production, causing spontaneous Ca2+ release from the SR, further contributing to arrhythmia. Adipokines in a diseased state cause a pro-inflammatory situation, including the recruitment and activation of macrophages, and change the pattern of gene expression. Several of the signalling pathways and receptors activated by adipokines are not yet identified.